PERINATAL HYPOXIC-ISCHEMIC BRAIN DAMAGE TREATMENT

This application claims the benefit, under 35 U.S.C. § 119(e) of United States Provisional Application Serial No. 60/527,056, filed December 3, 2003 and United States Provisional Application Serial No. 60/489,198, filed July 21, 2003, which are herein both incorporated by reference in their entirety.

Field of the invention

This invention relates to methods of treatment and prevention of perinatal hypoxic-ischemic brain damage.

BACKGROUND OF THE INVENTION

Perinatal hypoxic-ischemic brain damage is a major cause of acute mortality and chronic neurological morbidity in infants and children. The condition is particularly prevalent in low birth weight (premature) newborns. The basic cause is a reduction or interruption in the supply of oxygen to the brain of the fetus or newborn, commonly as a result of diminished placental perfusion, uteroplacental insufficiency or umbilical cord compression, and complications of delivery in the newborn, leading to asphyxiation. Pre-exposure to an inflammatory insult such as infection makes the infant more susceptible to hypoxic-ischemic brain injury. Between 20% and 50% of asphyxiated newborn infants who exhibit hypoxic-ischemic encephalopathy expire during the newborn period. Of the survivors, up to 25% exhibit permanent neuropsychological handicaps in the form of mental retardation, cerebral palsy, learning disability, or epilepsy. A fetus and newborn at risk for perinatal hypoxic-ischemic brain damage is readily recognized from experiences and events during late pregnancy and during

delivery. Moreover, the atrophy of the brain resulting therefrom continues over several days if not weeks. The brains of neonates that have experienced perinatal hypoxic-ischemic brain damage do not exhibit normal growth in subsequent years.

Infants born preterm are most susceptible to brain injury. They are prone to periventricular hemorrhage and periventricular leukomalacia. Both are caused by vascular dysfunction with impairment of blood flow to the injured region as an important part of the pathogenesis. The most common reason for premature onset of labor and delivery of a preterm infant is an inflammatory reaction of the placenta (chorioamnionitis) which in most cases is caused by infection. The inflammatory response in the placenta induces early labor. The infection of the placenta is also associated with elevated inflammatory cytokines in the amniotic fluid and the fetal brain. This systemic response to infection is referred to as the fetal inflammatory response and is associated with increased damage to the areas of the brain with lowest blood flow as well as an increase in the incidence of bronchopulmonary dysplasia , a chronic inflammatory process in the premature infants lung requiring prolonged hospitalization for mechanical ventilation and supplemental oxygen delivery. Full term infants who develop cerebral palsy have been shown to have elevated inflammatory cytokines when their umbilical cord blood samples are analyzed, supporting the notion that inflammation contributes to neonatal brain injury. The mechanisms whereby inflammation can contribute to injury is by activating endothelial neutrophil interactions, including the production of free radical species, and activating the coagulation pathway in blood vessels thereby impairing micro vascular function, red cell trafficking and tissue oxygen deliver. In addition peripheral macrophages (monocytes) are activated to produce inflammatory chemokines and cytokines that recruit inflammatory cells and induce them to release cytotoxic agents.

On occasion, it is necessary to conduct cardiac surgery on a neonate, for example surgical repair to the heart. This requires that the heart be bypassed and stopped, for up to thirty minutes, while surgical repairs are conducted on the heart. Hypoxic-ischemic brain damage in the neonate is a likely consequence of such a procedure. It can similarly arise from complications where the umbilical cord becomes entangled with the neonate's circulatory system during delivery, to cut off blood flow to the brain.

In short, situations where there is significant risk of development of hypoxic-ischemic brain damage in the neonate are readily recognizable, and not infrequent.

An animal model of perinatal hypoxic-ischemic brain damage has been developed and extensively studied, to gain insight into the underlying mechanisms of perinatal hypoxic-ischemic brain damage, and how tissue injury can be prevented or minimized through therapeutic intervention. The 7-day postnatal rat has a brain which is histologically similar to that of a 32-34 week gestation human fetus or newborn infant, in that the cerebral cortical neuronal layering is complete, the germinal matrix is involuting, and white matter as yet has undergone little myelination. In addition, the brain of the 12-13 day postnatal rat is roughly equivalent to that of the full-term newborn human infant (R.C. Vannucci, *et al.*, "Rat model of perinatal hypoxic-ischemic brain damage", Journal of Neuroscience Research, 55:158-163 (1999).

Currently there is no intervention based on controlled, randomized clinical trial evidence that can be recommended for the treatment of newborns with perinatal hypoxic –ischemic brain injury. In neonatal animal models various modalities have demonstrated that injury can be reduced if treatment is administered either before or after the insult. This shows that there is a therapeutic window for the appropriate agent. Strategies that impair the inflammatory component that accompanies and follows the

insult are protective in animal models. To date there have been limited clinical trials of potential pharmacologic agents because of the potential for toxicity especially in the newborn.

Arvin et. al., (Annals of Neurology Vol. 52, No. 1, July 2002, incorporated by reference in its entirety) report studies of the effectiveness of the antibiotic minocycline given as a single dose intraperitoneally, either immediately before or immediately after the hypoxic-ischemic injury in rats. They report a significant neuroprotective effect from minocycline and postulate that it is acting early in the neuronal death-promoting cascades to block necrosis and apoptosis.

Palmer, C. and R.L. Roberts, (Pedatr. Res., 1997. 41: 294A, incorporated by reference in its entirety) report promising results with delayed administration of allopurinol after cerebral hypoxia-ischemia brain injury in neonatal rats.

There remains a need for a safe, systemic neuroprotective therapy that can easily be used immediately on recognition that a hypoxic-ischemic injury in a perinatal patient is occurring or has occurred.

SUMMARY OF THE INVENTION.

The present invention provides treatments and compositions beneficially affecting perinatal hypoxic-ischemic brain damage.

The present invention is based upon the discovery that pharmaceutically acceptable bodies, such as liposomes, beads or similar particles, with presented phosphate-glycerol head groups may be used to treat perinatal hypoxic-ischemic brain damage.

In a preferred embodiment, the invention is directed to a method for inhibiting and/or reducing symptoms associated with perinatal hypoxic-ischemic brain damage in a perinatal patient comprising administering to the perinatal patient an

effective amount of phosphatidylglycerol (PG)-carrying bodies. Preferably, the bodies are of a size from about 20 nanometers (nm) to about 500 micrometers (μm) (as measured on its longest axis), more preferably from about 50 nanometers to about 1000 nanometers, and still more preferably from about 80 nanometers to about 120 nanometers.

Preferably, the bodies are essentially free of pharmaceutically active entities other than the phosphate-containing groups. Thus the bodies are not being used as carriers for pharmaceutical entities, but are active themselves, through the phosphate-glycerol groups. Preferably the phosphate-glycerol groups constitute 60% - 100% of the available phosphate-containing groups on the bodies, the balance thereof being inactive or active through a different mechanism. Thus the bodies described above may additionally comprise an inactive constituent surface group (such as phosphate-choline groups), and/or a constituent surface group which is active through another mechanism, (such as phosphate-serine groups from phosphatidylserine as described in Fadok *et al.*, International Publication WO 01/66785).

Such constituent surface groups, if present, should not constitute more than about 40% of the total functional surface groups, balance phosphate glycerol.

In another aspect this invention provides the use of phosphatidylglycerol (PG)-carrying bodies in the preparation of a medicament to inhibit and/or reduce the progression of perinatal hypoxic ischemic brain damage in a mammalian patient.

It is contemplated that the methods may be accompanied with one or more other modalities, such as, but not limited to, those described in Arvin, *et al.* and Palmer, *et al.* Administration in combination includes, for example, administration of the compositions described herein, prior to, during or after administration of the one or more other modalities. One of skill in the art will be able to determine the administration schedule and dosage.

BRIEF DESCRIPTION OF THE DRAWING

FIG. 1 is a diagrammatic illustration of a brain of a neonatal rat, indicating how measurements were taken in the experimental Example 2 described below.

DESCRIPTION OF PREFERRED EMBODIMENTS

1. Definitions

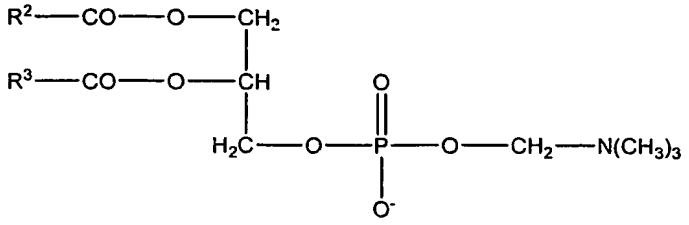
This section sets forth certain defined terms; other terms used herein are defined in context and/or have the meanings generally attributable to them in standard usage by those skilled in the art.

The term "biocompatible" refers to substances that, in the amount employed, are either non-toxic or have acceptable toxicity profiles such that their use *in vivo* is acceptable.

The terms "liposomes" and "lipid vesicles" refer to sealed membrane sacs, having diameters in the micron or sub-micron range, the walls of which consist of layers, typically bilayers, of suitable, membrane-forming amphiphiles. They normally contain an aqueous medium.

The term "pharmaceutically acceptable" has a meaning that is similar to the meaning of the term "biocompatible." As used in relation to "pharmaceutically acceptable bodies" herein, it refers to bodies of the invention comprised of one or more materials which are suitable for administration to a mammal, preferably a human, *in vivo,* according to the method of administration specified *(e.g.,* intramuscular, intravenous, subcutaneous, topical, oral, and the like).

The term "phosphate choline" refers to the group $-O-P(=O)(OH)-O-CH_2-CH_2-N^+-(CH_3)_3$, which can attached to lipids to form "phosphatidylcholine" (PC) as shown in the following structure:



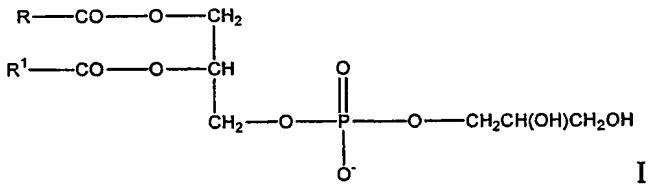
and salts thereof, wherein R^2 and R^3 are independently selected from C_1-C_{24} hydrocarbon chains, saturated or unsaturated, straight chain or containing a limited amount of branching wherein at least one chain has from 10-24 carbon atoms.

The term "phosphate-glycerol-carrying bodies" refers to biocompatible, pharmaceutically-acceptable, three-dimensional bodies having on their surfaces phosphate-glycerol groups or groups that can be converted to phosphate-glycerol groups, as described herein.

A "phosphate-glycerol group" is a group having the general structure: O-P(=O)(OH)-O-$CH_2CH(OH)CH_2OH$, and derivatives thereof, including, but not limited to groups in which the negatively charged oxygen of the phosphate group of the phosphate-glycerol group is converted to a phosphate ester group (*e.g.*, L-OP(O)(OR')(OR"), where L is the remainder of the phosphate-glycerol group, R' is -$CH_2CH(OH)CH_2OH$ and R" is alkyl of from 1 to 4 carbon atoms, or a hydroxyl substituted alkyl of from 2 to 4 carbon atoms, and 1 to 3 hydroxyl groups provided that R" is more readily hydrolyzed *in vivo* than the R' group; to a diphosphate group including diphosphate esters (*e.g.*, L-OP(O)(OR')OP(O)(OR")$_2$ wherein L and R' are as defined above and each R" is independently hydrogen, alkyl of from 1 to 4 carbon atoms, or a hydroxyl substituted alkyl of from 2 to 4 carbon atoms and 1 to 3 hydroxyl groups, provided that the second phosphate [-P(O)(OR")$_2$] is more readily hydrolyzed *in vivo* than the R' group; or to a triphosphate group including triphosphate esters (*e.g.*, L-OP(O)(OR')OP(O)(OR")OP(O)(OR")$_2$ wherein L and R' are defined as above and each

R" is independently hydrogen, alkyl of from 1 to 4 carbon atoms, or a hydroxyl substituted alkyl of from 2 to 4 carbon atoms and 1 to 3 hydroxyl groups provided that the second and third phosphate groups are more readily hydrolyzed *in vivo* than the R' group; and the like. Such synthetically altered phosphate-glycerol groups are capable of expressing phosphate-glycerol *in vivo* and , accordingly, such altered groups are phosphate-glycerol convertible groups within the scope of the invention. A specific example of a phosphate-glycerol group is the compound phosphatidylglycerol (PG), further defined herein.

"Phosphatidylglycerol" is also abbreviated herein as "PG." This term is intended to cover phospholipids carrying a phosphate-glycerol group with a wide range of at least one fatty acid chain provided that the resulting PG entity can participate as a structural component of a liposome. Chemically, PG has a phosphate-glycerol group and a pair of similar, but different fatty acid side chains. Preferably, such PG compounds can be represented by the Formula I:

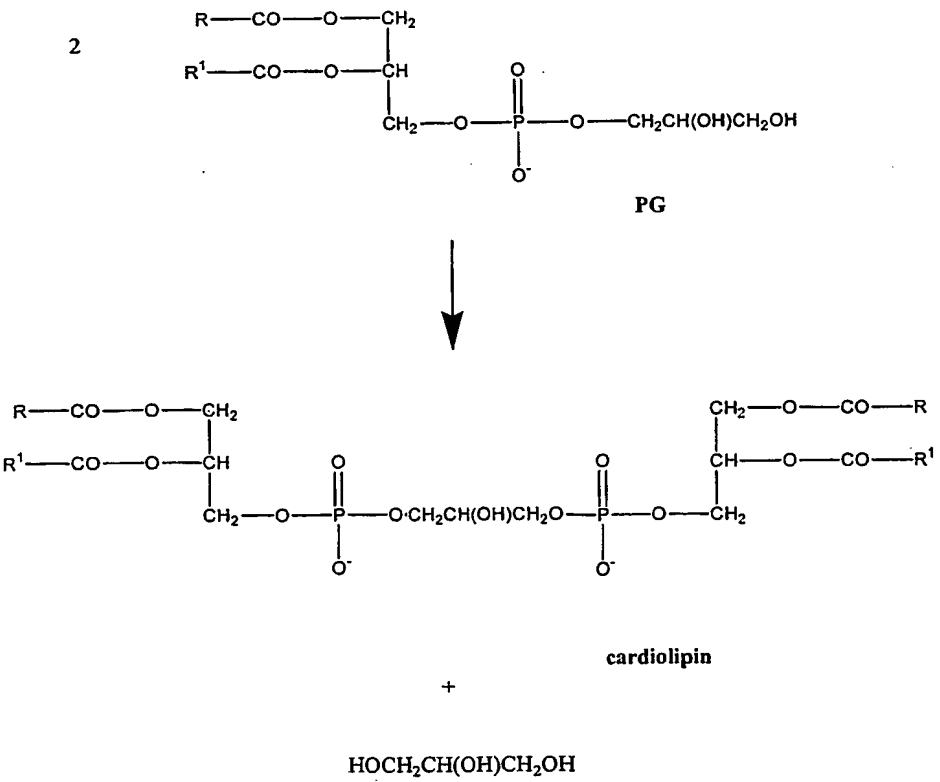


I

where R and R^1 are independently selected from C_1 -C_{24} hydrocarbon chains, saturated or unsaturated, straight chain or containing a limited amount of branching wherein at least one chain has from 10 to 24 carbon atoms. R and R^1 can be varied to include two or one lipid chain(s), which can be the same or different, provided they fulfill the structural function. As mentioned above, the fatty acid side chains may be from about 10 to about 24 carbon atoms in length, saturated, mono-unsaturated or polyunsaturated, straight-chain or with a limited amount of branching. Laurate (C12), myristate (C14), palmitate (C16),

stearate (C18), arachidate (C20), behenate (C22) and lignocerate (C24) are examples of useful saturated fatty acid side chains for the PG for use in the present invention. Palmitoleate (C15), oleate (C18) are examples of suitable mono-unsaturated fatty acid side chains. Linoleate (C18), linolenate (C18) and arachidonate (C20) are examples of suitable poly-unsaturated fatty acid side chains for use in PG in the compositions of the present invention. Phospholipids with a single such fatty acid side chain, also useful in the present invention, are known as lysophospholipids.

The term PG also includes dimeric forms of PG, namely cardiolipin, but other dimers of Formula I are also suitable. Preferably, such dimers are not synthetically cross-linked with a synthetic cross-linking agent, such as maleimide but rather are cross-linked by removal of a glycerol unit as described by Lehninger, *Biochemistry* and depicted in the reaction below:



Purified forms of phosphatidylglycerol are commercially available, for example, from Sigma-Aldrich (St. Louis, MO). Alternatively, PG can be produced, for example, by treating the naturally occurring dimeric form of phosphatidylglycerol, cardiolipin, with phospholipase D. It can also be prepared by enzymatic synthesis from phosphatidyl choline using phospholipase D. See, for example, U.S. Patent 5,188,951 (Tremblay *et al.*), incorporated herein by reference.

"PG-carrying bodies" are three-dimensional bodies, as described above, that have surface PG molecules. By way of example, PG can form the membrane of a liposome, either as the sole constituent of the membrane or as a major or minor component thereof, with other phospholipids and/or membrane forming materials.

The term "phosphatidylserine" or "PS" is intended to cover phosphatidyl serine and analogs/derivatives thereof.

In the context of the present invention, "three-dimensional bodies" refer to biocompatible synthetic or semi-synthetic entities, including but not limited to liposomes, solid beads, hollow beads, filled beads, particles, granules and microspheres of biocompatible materials, natural or synthetic, as commonly used in the pharmaceutical industry. Liposomes may be formed of lipids, including phosphatidylglycerol (PG). Beads may be solid or hollow, or filled with a biocompatible material. Such bodies have shapes that are typically, but not exclusively spheroidal, cylindrical, ellipsoidal, including oblate and prolate spheroidal, serpentine, reniform and the like, and have sizes ranging from 200 nm to 500 μm, preferably measured along the longest axis.

"Treatment" includes, for example, a reduction in the number of symptoms, a decrease in the severity of at least one symptom of the particular disease or a delay in the further progression of at least one symptom of the particular disease.

2. Phosphate-Glycerol-Carrying Bodies

This section describes various embodiments of phosphate-glycerol-carrying bodies contemplated by the present invention, including specific embodiments thereof. With the guidance provided herein, persons having requisite skill in the art will readily understand how to make and use phosphate-glycerol-carrying bodies in accordance with the present invention.

In the context of the present invention, phosphate-glycerol-carrying bodies refer to biocompatible, pharmaceutically-acceptable, three-dimensional bodies having on their surfaces phosphate-glycerol groups or groups that can be converted to phosphate-glycerol groups, as described herein.

a. Phosphate-Glycerol Groups

According to a general feature of the invention, phosphate-glycerol groups useful in the present invention have the general structure:

$$O\text{-}P(=O)(OH)\text{-}O\text{-}CH_2CH(OH)CH_2OH$$

Such phosphate-glycerol groups include synthetically altered versions of the phosphate-glycerol group shown above, and may include all, part of or a modified version of the original phosphate-glycerol group.

Preferably the fatty acid side chains of the chosen PG will be suitable for formation of liposomes, and incorporation into the lipid membrane(s) forming such liposomes, as described in more detail below.

More generally, without being limited to any particular theory, it is believed that phosphate-glycerol groups according to the present invention are capable of interacting with one or more receptors present in relevant brain tissue, such as the hippocampus. A specific example of a phosphate-glycerol group is the compound phosphatidylglycerol (PG), described above.

PG groups of the present invention, including dimers thereof, are believed to act as ligands, binding to specific sites on a protein or other molecule ("PG receptor")

and, accordingly, PG (or derivatives or dimeric forms thereof) are sometimes referred to herein as a "ligand" or a "binding group." Such binding is believed to take place through the phosphate-glycerol group -O-P(=O)(OH)-O-$CH_2CH(OH)CH_2OH$, which is sometimes referred to herein as the "head group," "active group," or "binding group," while the fatty acid side chain(s) are believed to stabilize the group and/or, in the case of liposomal preparations, form the outer lipid layer or bilayer of the liposome. More generally, again without being limited to any particular theory, it is believed that phosphate-glycerol groups, including PG are capable of interacting with one or more receptors in the brain and that such interactions may provide positive effects on synaptic transmission, and, by extension, memory, as described herein.

As noted above, analogues of phosphatidylglycerol with modified active groups, which also interact with PG receptors on the antigen presenting cells, through the same receptor pathway as PG or otherwise resulting in an anti-inflammatory reaction in the recipient body are contemplated within the scope of the term phosphatidylglycerol. This includes, without limitation, compounds in which one or more of the hydroxyl groups and/or the phosphate group is derivatized, or in the form of a salt. Many such compounds form free hydroxyl groups in vivo, upon or subsequent to administration and, accordingly, comprise convertible PG groups.

b. Formation of Phosphate-Glycerol Carrying Bodies

Phosphate-glycerol carrying bodies are three-dimensional bodies that have surface phosphate-glycerol molecules. This section will describe general and exemplary phosphate-glycerol carrying bodies suitable for use in the present invention.

Generally, phosphate-glycerol carrying bodies of the present invention carry phosphate-glycerol molecules on their exterior surfaces to facilitate *in vivo* interaction of the binding groups.

Three-dimensional bodies are preferably formed to be of a size or sizes suitable for administration to a living subject, preferably by injection; hence such bodies will preferably be in the range of 20 to 1000 nm (0.02-1 micron), more preferably 20 to 500 nm (0.02-0.5 micron), and still more preferably 20-200 nm in diameter, where the diameter of the body is determined on its longest axis, in the case of non-spherical bodies. Suitable sizes are generally in accordance with blood cell sizes. While bodies of the invention have shapes that are typically, but not exclusively spheroidal, they can alternatively be cylindrical, ellipsoidal, including oblate and prolate spheroidal, serpentine, reniform in shape, or the like.

Suitable forms of bodies for use in the compositions of the present invention include, without limitation, particles, granules, microspheres or beads of biocompatible materials, natural or synthetic, such as polyethylene glycol, polyvinylpyrrolidone, polystyrene, and the like; polysaccharides such as hydroxethyl starch, hydroxyethylcellulose, agarose and the like; as are commonly used in the pharmaceutical industry. Preferably, such materials will have side-chains or moieties suitable for derivatization, so that a phosphate-glycerol group, such as PG, may be attached thereto, preferably by covalent bonding. Bodies of the invention may be solid or hollow, or filled with biocompatible material. They are modified as required so that they carry phosphate-glycerol molecules, such as PG on their surfaces. Methods for attaching phosphate-glycerol in general, and PG in particular, to a variety of substrates are known in the art.

In addition to the various bodies listed above, the liposome is a particularly useful form of body for use in the present invention. Liposomes are microscopic vesicles composed of amphiphilic molecules forming a monolayer or bilayer surrounding a central chamber, which may be fluid-filled. Amphophlilic molecules (also referred to as

"amphiphiles"), are molecules that have a polar water-soluble group attached to a water-insoluble (lipophilic) hydrocarbon chain, such that a matrix of such molecules will typically form defined polar and apolar regions. Amphiphiles include naturally occurring lipids such as PG, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine, cholesterol, cardiolipin, ceramides and sphingomyelin, used alone or in admixture with one another. They can also be synthetic compounds such as polyoxyethylene alkyl ethers, polyoxyethylene alkyl esters and saccharosediesters.

Preferably, for use in forming liposomes, the amphiphilic molecules will include one or more forms of phospholipids of different head groups (*e.g.*, phosphatidylglycerol, phosphatidylserine, phosphatidylcholine) and having a variety of fatty acid side chains, as described above, as well as other lipophilic molecules, such as cholesterol, sphingolipids and sterols.

In accordance with the present invention, phosphatidylglycerol (PG) will constitute the major portion or the entire portion of the liposome layer(s) or wall(s), oriented so that the phosphate-glycerol group portion thereof is presented exteriorly, as described above, while the fatty acid side chains form the structural wall. When, as in the present invention, the bilayer includes phospholipids, the resulting membrane is usually referred to as a "phospholipid bilayer," regardless of the presence of non-phospholipid components therein.

Liposomes of the invention are typically formed from phospholipid bilayers or a plurality of concentric phospholipid bilayers which enclose aqueous phases. In some cases, the walls of the liposomes may be single layered; however, such liposomes (termed "single unilamellar vesicles" or "SUVs") are generally much smaller (diameters less than about 70 nm) than those formed of bilayers, as described below. Liposomes formed in accordance with the present invention are designed to be biocompatible,

biodegradable and non-toxic. Liposomes of this type are used in a number of pharmaceutical preparations currently on the market, typically carrying active drug molecules in their aqueous inner core regions. In the present invention, however, the liposomes are not filled with pharmaceutical preparation. The liposomes are active themselves, not acting as drug carrier.

Preferred PG-carrying liposomes of the present invention are constituted to the extent of 50% -100% by weight of phosphatidyl glycerol, the balance being phosphatidylcholine (PC) or other such biologically acceptable phospholipid(s). More preferred are liposomes constituted by PG to the extent of 65% -90% by weight, most preferably 70% -80% by weight, with the single most preferred embodiment, on the basis of current experimental experience, being PG 75% by weight, the balance being other phospholipids such as PC. Such liposomes are prepared from mixtures of the appropriate amounts of phospholipids as starting materials, by known methods. According to an important feature of the invention, PG-carrying bodies comprise less than 50%, preferably less than 40%, still preferably less than 25% and even still preferably less than 10% phosphatidyl serine.

The present invention contemplates the use, as PG-carrying bodies, not only of those liposomes having PG as a membrane constituent, but also liposomes having non-PG membrane substituents that carry on their external surface molecules of phosphate-glycerol, either as monomers or oligomers (as distinguished from phosphatidylglycerol), *e.g.*, chemically attached by chemical modification of the liposome surface of the body, such as the surface of the liposome, making the phosphate-glycerol groups available for subsequent interaction. Because of the inclusion of phosphate-glycerol on the surface of such molecules, they are included within the definition of PG-carrying bodies.

Liposomes may be prepared by a variety of techniques known in the art, such as those detailed in Szoka *et al.* (Ann. Rev. Biophys. Bioeng. 9:467 (1980)). Depending on the method used for forming the liposomes, as well as any after-formation processing, liposomes may be formed in a variety of sizes and configurations. Methods of preparing liposomes of the appropriate size are known in the art and do not form part of this invention. Reference may be made to various textbooks and literature articles on the subject, for example, the review article by Yechezkel Barenholz and Daan J. A. Chromeline, and literature cited therein, for example New, R. C. (1990), and Nassander, U. K., *et al.* (1990), and Barenholz, Y and Lichtenberg, D., *Liposomes: preparation, characterization, and preservation. Methods Biochem Anal.* (1988) 33:337-462.

Multilamellar vesicles (MLV's) can be formed by simple lipid-film hydration techniques according to methods known in the art. In this procedure, a mixture of liposome-forming lipids is dissolved in a suitable organic solvent. The mixture is evaporated in a vessel to form a thin film on the inner surface of the vessel, to which an aqueous medium is then added. The lipid film hydrates to form MLV's, typically with sizes between about 100-1000 nm (0.1 to 10 microns) in diameter.

A related, reverse evaporation phase (REV) technique can also be used to form unilamellar liposomes in the micron diameter size range. The REV technique involves dissolving the selected lipid components, in an organic solvent, such as diethyl ether, in a glass boiling tube and rapidly injecting an aqueous solution, into the tube, through a small gauge passage, such as a 23-gauge hypodermic needle. The tube is then sealed and sonicated in a bath sonicator. The contents of the tube are alternately evaporated under vacuum and vigorously mixed, to form a final liposomal suspension.

By way of example, but not limitation, Example 1 provides a detailed description of a method of preparing a PG-liposomal preparation for use in the present invention.

The diameters of the PG-carrying liposomes of the preferred embodiment of this invention range from about 20 nm to about 1000 nm, more preferably from about 20 nm to about 500 nm, and most preferably from about 20 nm to about 200 nm. Such preferred diameters will correspond to the diameters of mammalian apoptotic bodies, such as may be apprised from the art.

One effective sizing method for REV's and MLV's involves extruding an aqueous suspension of the liposomes through a series of polycarbonate membranes having a selected uniform pore size in the range of 0.03 to 0.2 micron, typically 0.05, 0.08, 0.1, or 0.2 microns. The pore size of the membrane corresponds roughly to the largest sizes of liposomes produced by extrusion through that membrane, particularly where the preparation is extruded two or more times through the same membrane. This method of liposome sizing is used in preparing homogeneous-size REV and MLV compositions. U.S. Patents 4,737,323 and 4,927,637, incorporated herein by reference, describe methods for producing a suspension of liposomes having uniform sizes in the range of 0.1-0.4 μm (100-400 nm) using as a starting material liposomes having diameters in the range of 1 μm. Homogenization methods are also useful for down-sizing liposomes to sizes of 100 nm or less (Martin, F. J. (1990) In: Specialized Drug Delivery Systems--Manufacturing and Production Technology, P. Tyle (ed.) Marcel Dekker, New York, pp. 267-316.). Another way to reduce liposomal size is by application of high pressures to the liposomal preparation, as in a French Press.

Liposomes can be prepared to have substantially homogeneous sizes of single, bi-layer vesicles in a selected size range between about 0.07 and 0.2 microns (70-

200 nm) in diameter, according to methods known in the art. In particular, liposomes in this size range are readily able to extravasate through blood vessel epithelial cells into surrounding tissues. A further advantage is that they can be sterilized by simple filtration methods known in the art.

Whilst a preferred embodiment of PG-carrying bodies for use in the present invention is liposomes with PG presented on the external surface thereof, it is understood that the PG-carrying body is not limited to a liposomal structure, as mentioned above.

3. Dosages, Modes of Administration and Utility

The phosphate-glycerol-carrying bodies of the invention may be administered to the patient by any suitable route of administration, including oral, nasal, topical, rectal, intravenous, subcutaneous and intramuscularly. At present, intramuscular administration is preferred, especially in conjunction with PG-liposomes.

The PG-carrying bodies may be suspended in a pharmaceutically acceptable carrier, such as physiological sterile saline, sterile water, pyrogen-free water, isotonic saline, and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations. Preferably, PG-carrying bodies are constituted into a liquid suspension in a biocompatible liquid such as physiological saline and administered to the patient in any appropriate route which introduces it to the immune system, such as intra-arterially, intravenously, intra-arterially or most preferably intramuscularly or subcutaneously.

The quantities of PG-carrying bodies to be administered will vary depending on the identity and characteristics of the patient. It is important that the effective amount of PG-bodies is non-toxic to the patient. The most effective amounts are unexpectedly small. When using intra-arterial, intravenous, subcutaneous or

intramuscular administration of a liquid suspension of PG-carrying bodies, it is preferred to administer, for each dose, from about 0.1-50 ml of liquid, containing an amount of PG-carrying bodies generally equivalent to 10% -1000% of the number of leukocytes normally found in an equivalent volume of whole blood or the number of apoptotic bodies that can be generated from them. Generally, the number of PG-carrying bodies administered per delivery to a patient is in the range from about 500 to about 2.5×10^{12} (about 260 nanograms by weight), preferably from about 5,000 to about 500,000,000, more preferably from about 10,000 to about 10,000,000, and most preferably from about 200,000 to about 2,000,00

According to one feature of the invention, the number of such bodies administered to an injection site for each administration is believed to be a more meaningful quantization than the number or weight of PG-carrying bodies per unit of patient body weight. Thus, it is contemplated that effective amounts or numbers of PG-carrying bodies for small animal use may not directly translate into effective amounts for larger mammals on a weight ratio basis.

It is contemplated that the PG-carrying bodies may be freeze-dried or lyophilized to a form which may be later resuspended for administration. This invention therefore also includes a kit of parts comprising lyophilized or freeze-dried PG- carrying bodies and a pharmaceutically acceptable carrier, such as physiological sterile saline, sterile water, pyrogen-free water, isotonic saline, and phosphate buffer solutions, as well as other non-toxic compatible substances used in pharmaceutical formulations. Such a kit may optionally provide injection or administration means for administering the composition to a subject.

It is contemplated that the methods of the invention will be useful for treating perinatal patients. As used herein, the term "perinatal patient" refers to infants

who are at risk of or showing signs of development of perinatal hypoxic-ischemic brain damage. These perinatal patients may be treated prior to or post birth.

A preferred manner of administering the pharmaceutically acceptable bodies to the patient is by injection, administered intramuscularly or subcutaneously to the newborn at risk of or showing signs of development of perinatal hypoxic-ischemic brain damage. One or more injections may be administered, on a daily or twice daily basis, for the first several days of life, or on a different schedule according to the observations of the attending clinician. Since the brain atrophy resulting from the hypoxic-ischemic insult can continue for an extended period, the treatment according to the preferred aspects of the invention may beneficially be continued for several months.

A pre-term infant is at risk of developing brain injury for several weeks after birth, depending on the level of prematurity, so that prophylactic administration for weeks may be necessary for this population.

It is also within the scope of the present invention to administer the pharmaceutically acceptable bodies described above to the mother, prior to delivery and birth of the neonate or neonatal infant. Typically a neonatal infant is an infant born prematurely or otherwise, under 4 weeks old. As noted above, mothers at risk of giving birth to a premature neonate or a neonate likely to experience hypoxic-ischemic brain damage, are often recognizable. Any method of administration of the compositions of the invention that ensures that an effective amount of the composition reaches the placenta and crosses the placental barrier can be used on the mother. Intramuscular injection is preferred. Any mother giving birth to a pre-term baby is a candidate for treatment according to this invention.

It is postulated that, in many embodiments of the present invention, pharmaceutically acceptable bodies comprising the phosphate-glycerol head groups as

binding groups on their surface are acting as modifiers of the patient's immune system, in a manner similar to that of a vaccine. Accordingly they are used in quantities and by administration methods to provide a sufficient localized concentration of the bodies at the site of introduction. Quantities of such bodies appropriate for immune system modification may not be directly correlated with body size of a recipient and can, therefore, be clearly distinguished from drug dosages, which are designed to provide therapeutic levels of active substances in a patient's bloodstream and tissues. Drug dosages are accordingly likely to be much larger than immune system modifying dosages.

The correlation between weights of liposomes and numbers of liposomes is derivable from the knowledge, accepted by persons skilled in the art of liposomal formulations, that a 100 nm diameter bilayer vesicle has 81,230 lipid molecules per vesicle, distributed approximately 50:50 between the layers (see Richard Harrigan – 1992 University of British Columbia PhD Thesis "Transmembrane pH gradients in liposomes (microform): drug-vesicle interactions and proton flux", published by National Library of Canada, Ottawa, Canada (1993); University Microfilms order no. UMI00406756; Canadiana no. 942042220, ISBN 0315796936). From this one can calculate, for example, that a dose of 5×10^8 vesicles, of the order of the dose used in the specific *in vivo* examples below, is equivalent to 4.06×10^{13} lipid molecules. Using Avogadro's number for the number of molecules of lipid in a gram molecule (mole), 6.023×10^{23}, one determines that this represents 6.74×10^{-11} moles which, at a molecular weight of 729 for PG is approximately 4.92×10^{-8} gm, or 49.2 nanograms of PG for such dosage. For a dose of 6×10^5 vesicles , of the order of the dose used in the specific *in vivo* examples below, using animals of average weight 15 grams, the corresponding calculation gives a weight of 5.89×10^{-1} gm, or 0.059 nanograms.

The quantities of the pharmaceutically acceptable bodies to be administered are very small. Preferably, the effective amount of pharmaceutically acceptable bodies is non-toxic to the patient, and is not so large as to overwhelm the immune system. When using intra-arterial, intravenous, subcutaneous or intramuscular administration of a sterile aqueous suspension of pharmaceutically acceptable bodies, it is preferred to administer, for each dose, from about 0.1-50 ml of liquid, containing a number of bodies in the range from about 500 to about 2.5×10^9 (<250 ng of bodies, in the case of liposomes, pro-rated for density differences for other embodiments of bodies), more preferably from about 1,000 to about 1,500,000,000, even more preferably 10,000 to about 100,000,000, and most preferably from about 200,000 to about 2,000,000.

Since the pharmaceutically acceptable bodies are believed to be acting, in the process of the invention, as immune system modifiers, in the nature of a vaccine, the number of such bodies administered to an injection site for each administration may be a more meaningful quantitation than the number or weight of bodies per unit of patient body weight. For the same reason, it is now contemplated that effective amounts or numbers of bodies for small animal use may not directly translate into effective amounts for human newborns on a weight ratio basis.

EXAMPLES

The following examples are intended to illustrate methods for preparing therapeutic compositions of the present invention and exemplary treatment results. The examples are in no way intended to limit the scope of the invention.

Example 1

Preparation of Liposomes

A dry mixture ("Lipid Premix") was prepared, consisting of semi-synthetic POPG (1-palmitoyl-2-oleoly-sn-glycero-3-phosphoglycerol sodium salt), 3 parts by mass, and POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), 1 part by mass.

The POPC ingredient was prepared from DPPC (dipalmitoyl-sn-glycero-3-phosphocholine) which was purified from soybean and enzymatically hydrolyzed with porcine pancreas phospholipase A2 (E.C. 3.1.1.4) to generate monopalmitoyl phosphatidylcholine (MPPC). The MPPC was acylated with oleic acid to generate POPC. The POPC was recovered and further purified by liquid phase chromatography to a purity of not less than 98%. The purified material was dried, dissolved in appropriate solvent (ethanol, t-butanol or chloroform), filtered through 0.22 micron filter and subsequently dried in a clean room.

The POPG ingredient was prepared from POPC. The POPC was dissolved in a suitable solvent (ethanol, t-butanol or chloroform) and incubated with excess glycerol in the presence of recombinant phospholipase D (E.C. 3.1.4.4). POPG was recovered and purified by liquid phase chromatography to a purity of not less than 98%. The material was dried, dissolved in appropriate solvent (ethanol, t-butanol or chloroform), filtered through 0.22 micron filter and subsequently dried in a clean room.

POPG and POPC were dissolved at a ratio of 3:1 by mass in t-butanol, followed by filtration (0.22 micron) and drying in a clean room, to form the Lipid Premix. These steps were performed for the Applicants by Lipoid GmbH, Frigensr 4, Ludwigshafen.

The Lipid Premix was hydrated with phosphate buffered saline (PBS, pH 7.0, sterilized by filtration through a 0.22 micron sterilizing filter). A suspension of

multilamellar vesicles (MLV's) formed. The suspension was passed through polycarbonate filter (100 nm pore size) under pressure, generating unilamellar vesicles of about 100 nm in diameter. Vesicle size was verified, in-process, using a Quasi-Elastic Light Scattering (QELS) analysis. The suspension of unilamellar vesicles (liposomes) was immediately removed to a class 1,000 clean room, where it was redundantly filtered (0.22 micron) and filled into vials (1 mL per 2 mL amber vial) in a class 100 laminar flow hood. The vials were backfilled with nitrogen and sealed with butyl rubber stopper and aluminium crimp seals.

Example 2

Animal Model Testing

The Rice-Vannucci model of hypoxic ischemic brain injury in the neonatal rat is used for testing the formulations in the present invention – see Rice, J.E., R.C. Vannucci and J.B. Brierley, "The influence of immaturity on hypoxia-ischemic brain damage in the rat", Ann. Neurol., 1981.9: p.113-141. There is an evolving inflammatory reaction in the injured brain following hypoxia-ischemia in the immature rat pup model. Inflammatory cytokines such as IL-1β increase in the first 24 hours of recovery (1). The inflammatory reaction is characterized by an increase in activated microglial cells, expression of inflammatory cytokines and chemokines, and even the influx of CD4 lymphocytes after a few days. Prior exposure to endotoxin (LPS) increases the rats' susceptibility to brain damage following hypoxic-ischemia (2). Also, there is a delayed component of brain cell death (mainly by apoptosis) that continues for at least a week after the initial insult (3, 4). There is evidence of foci of injury surrounded by microglial for over 6 months in the immature rat pup model used here (5).

In order to demonstrate modulation of the inflammatory response following the insult in an attempt to reduce injury in accordance with a preferred embodiment of the invention, the following experiments are conducted.

Timed pregnant rats (2 per week) were allowed to deliver. On day one of life, *88* rat pups were numbered sequentially and assigned to one of two groups. Also on day 1 of life, one group was injected subcutaneously, at a fold of skin at the back of the neck, with 75% PG/ 25% PC liposomes, and the other group similarly injected, on the same schedule, with saline as control. Each injection consisted of a volume of 0.01 ml, per gram of body weight of the pup. The concentration of the liposomes in the suspension was 3 x 10^6 liposomes per ml, so that each pup received $3x10^4$ vesicles per gram body weight in each injection. The treatments (injections) were repeated on day 2 of life, day 6 of life and day 8 of life.

On day 7 of life, the rats were subjected to hypoxic-ischemic insult. For this purpose, the rats were anesthetized with Halothane/nitrous oxide and the right common carotid artery was permanently ligated with silk. After three hours recovery, the pups were placed in glass jars resting in a temperature controlled incubator and exposed to a hypoxic gas mixture (8% oxygen, balance nitrogen) for 2.25 hrs. Then the pups were returned to room air. This insult produces atrophy to the right hemisphere of the brain, while leaving the left hemisphere virtually intact. Following the hypoxic interval, the jars were opened to room air, and the pups were returned to their dams and returned to the animal care facility to recover for *21* days. Then they were sacrificed with a lethal dose of pentobarbital and their brains carefully removed intact and immersed in fixative.

Gross Assessment

After a week in fixative, the brains were removed from fixative and examined intact, to arrive at a gross assessment. The brains were examined by two

experienced investigators who were unaware of the identity of the treatment group. They allocated the brains an injury score (0-4) based on the difference in size of the ipsilateral (right) vs. contralateral (left) hemisphere. The examiners came to a consensus and agreed on each score, using the following criteria:

0 = no injury; no difference in size between hemispheres;

1 = mild injury; about 15% reduction in R hemisphere size;

2 = mild injury, about 15 – 30% atrophy, no cavitating injury;

3 = moderate injury, >30% atrophy, cavitating lesion with some preservation of R hemisphere;

4 = severe injury, almost complete destruction of the R hemisphere.

There were 42 rat brains from liposome-treated animals and 37 rat brains from saline treated animals, that had survived the hypoxia and the *3* weeks recovery. The brains from liposome treated rats had lower categories of injury that the brains from saline treated rats (P=0.0002, Mantzel-Hantzel test). Of the brains from saline treated rats, there were four with category 4 injury and eight with category 3 injury. None of the brains from liposome treated animals showed category 4 injury, and only three showed a category 3 injury, despite the fact that this was a larger group than the saline treated animals.

Morphometric analysis of brain atrophy

The fixed brains were cut with a blade into three 2 mm thick coronal slices from the mid section of the brain region that represented the area of maximum injury, as indicated on the accompanying Figure. This shows diagrammatically the rat pup brain 10 with its left hemisphere 12 and its right hemisphere 14. The area of hypoxic-ischemic damage 16 is in the right hemisphere 14. The slices were made by cutting coronal to the base of the brain, to obtain slice 2, slice 3 and slice 4.

A digital image was made of the three sections. The area of each hemisphere was measured using NIH image software. The percentage atrophy of the right hemisphere was calculated using the formula:

$$\frac{\text{Area L} - \text{Area R} \times 100}{\text{Area L}}$$

The atrophy in each slice 2, 3 and 4 was calculated and the results averaged to show the average atrophy. The results from the treatment group were compared with those from the saline, control group.

Brains from the saline treated rats had approximately double the amount of atrophy compared with those from the liposome treated rats. This was consistent for each slice. For all the slices, the average amount of atrophy for the saline treated group was 33.6 ± 21.7% (mean ± SD), while the liposome treated group had only 16.8 ±14.4%, a statistically significant difference ($p < 0.001$, Mann Whitney U. test). Thus liposome treatment according to this embodiment of the invention reduced atrophy by approximately 50%. The results are given in the Table below.

Table – Percentage Right Hemisphere Atrophy Comparisons.

	Saline, n=37	Liposome, n=42	Mann-Whitney U
Slice 2	27.8±18.8	14.2±14.8	0.001
Slice 3	35.4 ±24.0	17.14±13.9	<0.001
Slice 4	37.5±23.6	19.01±15.37	<0.001
Average	33.6±21.7	16.8±14.4	<0.001

This indicated a clinically significant reduction in injury consequent upon the hypoxia-ischemia insult in this model, and highlights the potential for the use of the invention in the treatment of perinatal hypoxic-ischemic brain damage.

References

1. Bona , E., *et al.*, Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. Pediatr Res, 1999. 45(4 Pt 1): p. 500-509.

2. Eklind, S., *et al.*, Bacterial endotoxin sensitizes the immature brain to hypoxic-ischaemic injury. Eur J Neurosci, 2001. 13(6): p.1931-8.

3. Northington, F.J., *et al.*, Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. J Neurosci, 2001. 21(6): p. 1931-8.

4. Nakajima, W., *et al.*, Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. J. Neurosci, 2000 20(21): p. 7994-8004.

5. Palmer, C., *et al.*, Iron containing "plaques" developing the brains of rats months after neonatal hypoxic-ischemic brain injury. Pediatr Res, 1998. 43: p. 322A.